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Morphological Features of Embryogenesis in *Drosophila melanogaster* Infected with a Male-killing Spiroplasma

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ABSTRACT—Morphological studies were conducted with *Drosophila melanogaster* embryos maternally infected with the 'sex-ratio' organism, a species of Spiroplasmas which specifically kill male zygotes. Abnormalities occurred at the stages from as early as cleavage or blastoderm to morphogenetic movements. The most remarkable feature was defective organization of the ventral nervous tissues, a result which fits well with that of previous mosaic analysis showing that the focal region of infection localizes at the ventral midline of the blastoderm.

INTRODUCTION

It has been rather long since the finding of an abnormal 'sex-ratio' (SR) condition in *Drosophila*, where females exceed males in number disturbing normal sex ratio of 1:1 (see ref. [1] for review). This trait is cytoplasmically inherited from generation to generation through ovaries and eggs [1–3]. Some of SR conditions have been proposed to be brought about by infectious, parasitic microorganisms [1] and finally it was demonstrated that the causal agent is a species of mycoplasma having a spiral form with a dimension of 4 to 8 μm in length and 0.1 to 0.2 μm in diameter [2]. This type of organism, although belongs to Spiroplasmas (cf. [3]), has often been called the sex-ratio organism and will be abbreviated as SRO. The SROs are found in the hemolymph of females of infected *Drosophila* strains and can be transferred to females of the same or other species by intra-abdominal injection, making non-SR strains into SR-ones [4]. Males are absent in the transferred as well as in the original SR strains, and this characteristic is accountable almost wholly through mortality during embryogenesis [1].

We previously analyzed gynandromorph survivals in *D. melanogaster* having a maternally infected SRO line and suggested that the focus of action of the SRO locates in the close vicinity to the ventral midline [5]. In the present study, we observe by light and electron microscopes the SRO-infected zygotes of *D. melanogaster* at their embryonic stages and describe that the most markedly affected organ is in fact the ventral nervous system.

MATERIALS AND METHODS

Collection of eggs

The Oregon-R strain of *D. melanogaster* infected with the SRO of the *D. nebulosa* origin was used, since this heterologous combination has offered a stable and convenient investigation system [5]. Female adults of the infected Oregon-R strain aged 7 to 10 days after emergence were crossed to young males of Oregon-R strain and fed on yeast-enriched food for 3 days at 25°C. Eggs were deposited at 25°C onto filter paper, which had been dipped in a suspension of yeast and placed on an agar plate. The eggs were collected at intervals and washed with distilled water for

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several times to remove the debris of yeast and then treated outright or incubated until desired age. As a control, normal Oregon-R eggs were processed equally as described above.

Light microscopic observation of intact embryos

Eggs collected were allowed to develop for 24 hr at 25°C. Then they were counted for hatchability and, after deprived of the chorions by brief treatment with 3% sodium hypochlorite, were observed for the terminal abnormality under a phase-contrast microscope.

Another series of eggs were observed for the embryogenesis continuously. The chorions were removed as above. Then, young embryos undergoing cleavage mitosis were selected by the method accordind to Bownes [6], submerged in the *Drosophila* Ringer solution on a glass depression slide to allow development and subjected to inspection individually under a phase-contrast microscope.

Light microscopic observation of sectioned embryos

Dechorionated embryos at desired age were fixed in a mixture of 4 parts 95% ethanol, 1 part 50% acetic acid and 1 part formalin or 25% glutaraldehyde in 0.1 M phosphate buffer at pH 6.8 shaken with octane for 2–5 min. Vitelline membranes were removed with fine tungsten needles in the aqueous phase of the fixing mixture [7]. The fixed embryos were washed with 70% alcohol, dehydrated with a series of ethanol and finally with n-butyl alcohol. To facilitate observation, the eggs were stained *in toto* with 1% Eosine dissolved in 95% ethanol in the course of dehydration. The stained and dehydrated embryos were embedded in a solution of butoxyethanol and glycol methacrylate (Polysciences Inc., a JB-4 embedding kit) [8], sectioned with a Porter-Blum Sorvall MT-1 ultramicrotome at 2 μm thickness, and stained with Giemsa solution (diluted with 0.12 M phosphate buffer, pH 7.4) for 20 min. The stained materials were mounted in Permount and observed under a compound microscope.

Electron microscopic observation

Eggs were dechorionated, fixed and removed from vitelline membranes as described above. The fixation was continued after removing vitelline membranes to make the total time of fixation 4 hr. The specimens were washed with sucrose-phosphate buffer of the same osmolarity as that of the fixative and allowed to stand overnight at 0°C, rapidly dehydrated in ethanol series and embedded in Epon 812. Embryos were sectioned at 60–150 nm thickness with a Porter-Blum Sorvall MT-1 ultramicrotome and stained with uranyl acetate and lead mixture. A JEM 7A type electron microscope was used for observation. Ovaries taken from female adults of the infected Oregon-R strain were fixed, embedded and sectioned followed by the observation for ultrastructure as above.

RESULTS

Hatchability and terminal abnormalities

The eggs of *D. melanogaster* maternally infected with the SROs from *D. nebulosa* had the hatchability of about 50 % (Table 1). The resulting adults were found to be all females, and thus the unhatched embryos should mainly be males which were killed during embryogenesis. About one fifth of the unhatched eggs showed no sign of development as seen in Figure 1 a and were white in color, indicating early lethality or block of fertilization (Table 1). The majority of the unhatched eggs exhibited somewhat developed features (Fig. 1 b and c) and were brown in color, indicating late lethality (Table 1). Almost all the embryos of the late lethal group showed a common characteristic feature of morphology, with about one third of yolk mass at the posterior part and two thirds of cellular mass at the anterior part (see Fig. 1 b and c). The late lethal embryos had larval tissues in disordered arrangements at the cellular region (see below). Arrows show the yolk mass.

The results from continuous observation of embryos indicated that cessation of development occurred at the stages from cleavage mitosis to the early morphogenetic movements. The embryos which were abnormal at the cleavage stage never developed beyond the blastokinesis stage and later broke down as non-brown lethal embryos. These

TABLE 1. Hatchability and terminal phase of eggs of *D. melanogaster* infected with sex-ratio organisms[a]

Strain	The number and percentage of eggs			Total
	hatched	unhatched		
		Early lethals or unfertilized[b]	Late lethals[b]	
SRO-infected	431[c] (48%)	80[d] (9%)	383[d] (43%)	894
Control	581[c] (93%)	26[d] (4%)	18[d] (3%)	625

a: Females of Oregon-R strain of *D. melanogaster* infected with the *D. nebulosa* SRO (or of the uninfected control) were aged 7 to 10 days after eclosion and crossed to males from Oregon-R. Eggs were collected at 1 or 2 hr intervals, incubated for 24 hr at 25°C, then the hatched individuals were counted and types of terminal abnormal embryos were estimated.

b: See text and Fig. 1.

c: The ratios of individuals developed into adults were about 94% (all females) for the SR-strain and about 45% females and 45% males for the control.

d: One might argue that the embryos of the SR-strain contain spontaneously occurring lethals as deduced from these data. Although this possibility could not be ruled out, the overall observation presented in this study may be reasonable because the SR-condition is clear-cut as shown in c and because we found the most common features out of several hundreds of specimens.

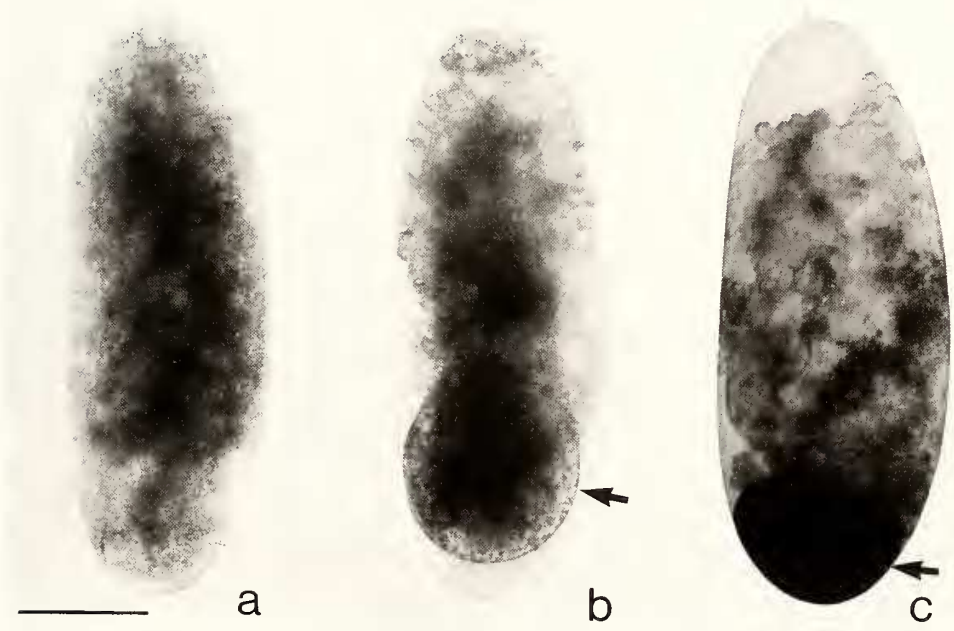

FIG. 1. Typical abnormal embryos infected with sex-ratio organisms. Dechorionated eggs were observed under a phase-contrast microscope as described in Materials and Methods. a) An early lethal embryo which stopped development at cleavage mitosis (aged 5 to 6 hr after oviposition). b) A late lethal embryo showing cellular mass at the anterior part and unused yolk mass at the posterior end (aged 20 to 22 hr after oviposition). c) A late lethal embryo with distinct larval tissues (aged 24 hr after oviposition). Scale, 100 μm.

were typical early lethal embryos like that shown in Figure 1 a. Some embryos indicated their abnormality as the retardation of blastoderm formation. These embryos in time become anomalous in appearance and finally indistinguishable from those which showed their first abnormality during early morphogenesis. These were typical late lethal embryos like those shown in Figure 1 b and c.

Features of dying embryos

Samples taken at various times of embryogenesis were sectioned and inspected after staining for histology in relation to the stage or state the respective embryos were in (Table 2). During the first 2 hr of development after oviposition, difference between the SRO-infected embryos and the normal ones was seldom detected, and at 2 hr about 70% of the individuals were at the syncytial blastoderm stage. At the 3rd hr, infected samples showed significant delay of development, about 50% of which were still at the syncytial blastoderm stage and about 10% at cleavage mitosis (most of the normal embryos were already at or beyond the cellular blastoderm stage).

At 3 to 5 hr after oviposition, the delay became greater (data not shown), and some of abnormal embryos began to break down exhibiting energid-like cytoplasmic islands which seemed to begin to fall apart. These energid-like structures consisted of disturbed fibers (remnants of spindle fibers and/or chromosomes?) as also seen in the SRO-infected *D. willistoni* embryos [9]. Some of other infected embryos were again at the cleavage stage or at the syncytial to cellular blastoderm stages.

As to the embryos which showed the first sign of abnormality at later stages beyond gastrulation, the deviation from the normal course of development was expressed as the appearance of unusual, necrotic type of cells. These cells were characterized by their strong stainability of the cytoplasm (dark blue with Giemsa), clear and large nucleus with a ring-shaped nucleolus-like structure and roundness of the cell surface under a light microscope. An electron microscopical appearance of this type of cells is seen in Figure 2. Autoradiographical study has suggested that these cells are inactive in RNA synthesis and protein synthesis (Tsuchiyama-Omura, unpublished data). They may therefore be a sort of retrograding cells. These occurred mainly in the neuroblast region 5–6 hr of the development (Fig. 3). As the SRO-infected embryos develop, such cells gradually increase in number also in the hypodermis, midgut or other tissues as well and coming to make a cluster.

More and more infected embryos were categorized as abnormal, even when they still did not reach the terminal features. At 9 hr after oviposition the cells constructing the hypodermis, neuroblast, ventral nervous system and mesodermal structures seemed to be somewhat disorderly intermingled with each other. During the succeed-

TABLE 2. Comparison of development between infected and control embryos[a]

Time of observation	Strain	The number and percentage of eggs at the stage or state of					Total
		unfertilized	cleavage mitosis	syncytial blastoderm	cellular blastoderm	early gastrulation	
1±0.5 hr	SR	1 (1%)	68 (94%)	3 (4%)	0	0	72
	Control	1 (3%)	26 (72%)	3 (8%)	4 (11%)	2 (6%)	36
2±0.5 hr	SR	5 (10%)	7 (14%)	33 (66%)	5 (10%)	0	50
	Control	1 (4%)	5 (18%)	19 (68%)	3 (11%)	0	28
3±0.5 hr	SR	4 (4%)	7 (8%)	46 (52%)	23 (26%)	9 (10%)	89
	Control	4 (7%)	0	8 (13%)	31 (51%)	18 (30%)	61

a: Females of SRO-infected or control Oregon-R strains were crossed as described in Table 1 and eggs were collected at indicated times, sectioned and inspected light microscopically. There were significant differences between SRO-infected and control embryos by means of χ^2-test in the 3rd-hr samples.

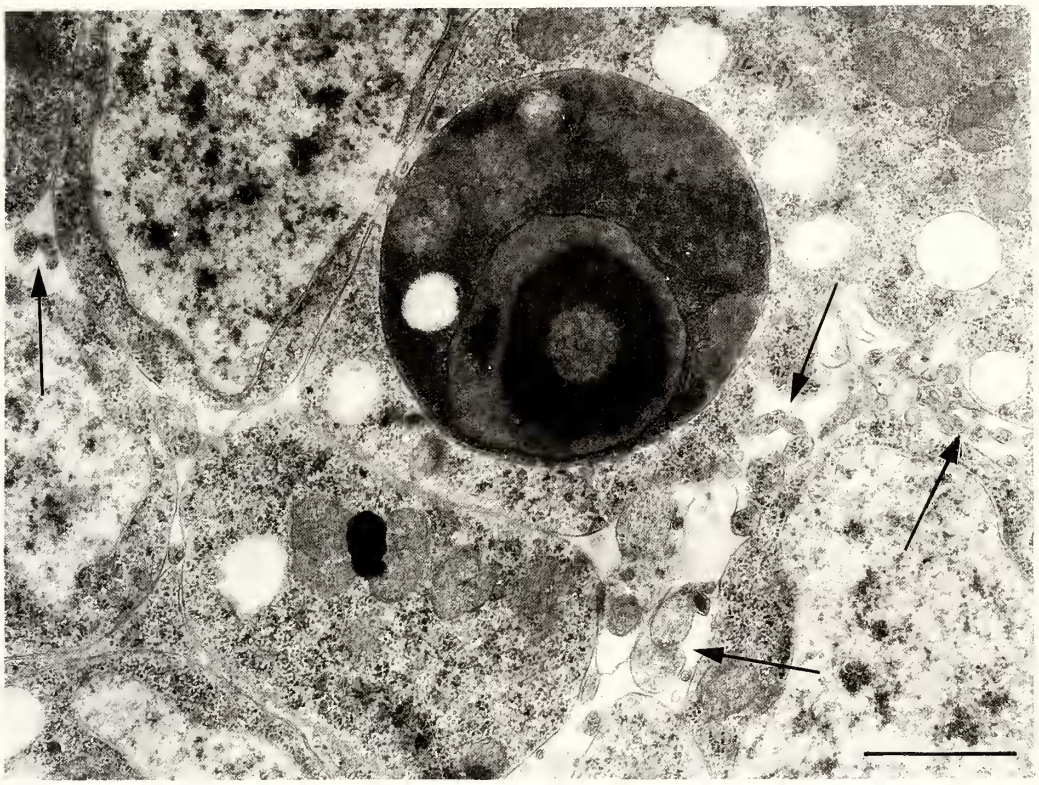

FIG. 2. Electron micrograph of a part of an embryo infected with sex-ratio organisms. An late lethal embryo at 10 hr old was taken to show a necrotic, retrograding cell (a dense and round cell in the center). Arrows point the SRO-like structures seen in the intercellular areas with low electron density. Scale, 1 μm.

ing periods the hypodermic and mesodermal tissues followed the normal course of development, that is, the hypodermis thinned out dorsally at about 10 hr (Fig. 4), and the somatic visceral and pharyneal muscles appeared from this time on. The salivary gland with or without contents (mucoprotein?) could also be found in most of the abnormal embryos. On the other hand, the region of ventral nervous system in SRO-infected embryos was shorter than normal even before the onset of shortening and little nerve fibers were found. Nevertheless, the brain was clearly identified at the ordinary place with normal configuration in most individuals. The fore- and hindgut looked quite normal, but the mid-ventral part of the midgut was disorganized having retrograding cells. Some of the latter tend to fall into the yolk mass which was abnormally concentrated in the center of an embryo (Fig. 4). The hypodermis began to show the sign of cuticle secretion after the 12th hour which is the normal time course of uninfected embryos. The gonad-like structures were occasionally observed.

During the 11–14th hr after oviposition, the yolk mass began to extrude through the feeble and disorganized ventral nervous system leaving other tissues behind. After this incident, abnormal embryos could be easily recognized by the typical terminal phenotype of SRO-infected embryos. Following the extrusion of yolk, tissues seemed to develop autonomously; i.e. embryos from later samples showed more developed structures of tissues, although their interorganic arrangement was completely disturbed. This disturbance occurred increasingly as the time went, changing most of the abnormal embryos into the typical late lethal ones by the 20–22nd hr after oviposition. Figure 5 illustrates such features. The unabsorbed yolk

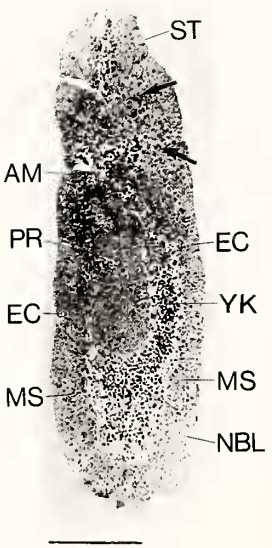

FIG. 3. Features of a 5 to 6 hr old embryo infected with sex-ratio organisms. A specimen was cut, stained and observed under a compound microscope as described in Materials and Methods. Arrows show necrotic cells. AM, amnion; EC, ectoderm; MS, mesoderm; NBL, neuroblast; PR, proctodaeum; ST, stomodaeum; YK, yolk mass. Scale, 100 μm.

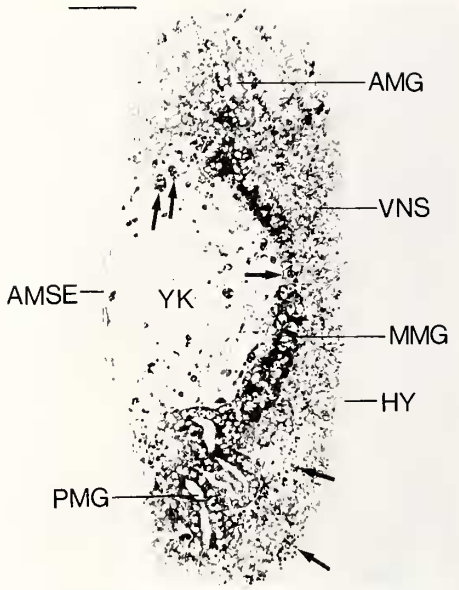

FIG. 4. Features of a 10 hr old embryo infected with sex-ratio organisms. A specimen was cut, stained and observed as described in Fig. 3. Arrows indicate retrograding cells in the midgut, hypodermis etc. AMG, anterior midgut; AMSE, amnioserosa; HY, hypodermis; MMG, middle midgut; PMG, posterior midgut; VNS, ventral nervous system; YK, yolk mass. Scale, 50 μm.

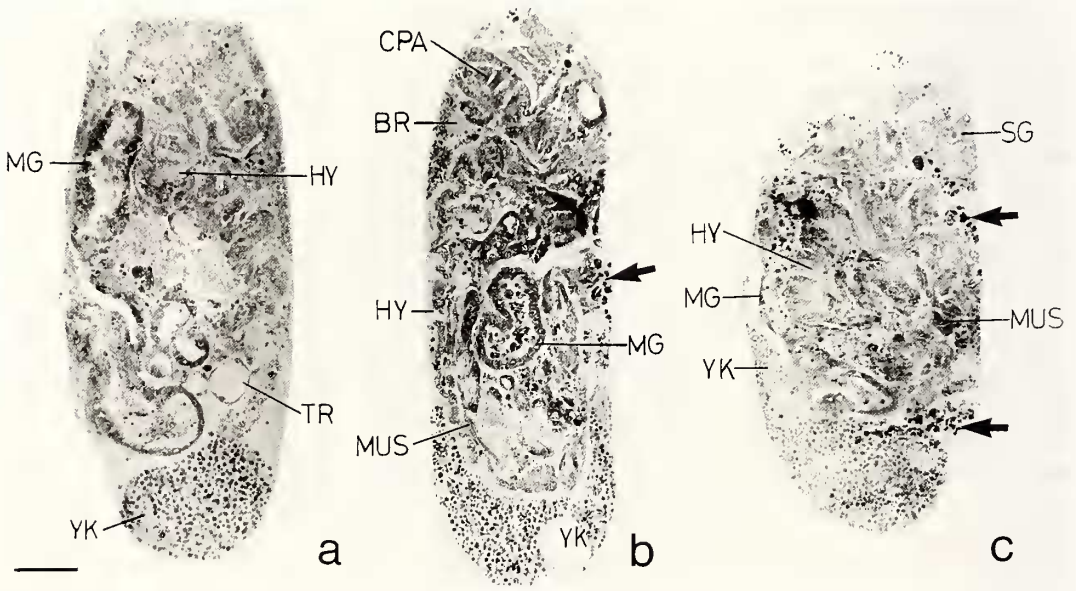

FIG. 5. Features of 22 to 25 hr old embryos infected with sex-ratio organisms. Specimens were cut, stained and observed as described in Fig. 3. a, b and c are from different individuals to show variety in arrangement of larval tissues from embryo to embryo. Arrows indicate necrotic cells. BR, brain; CPA, cephalopharyngeal apparatus; HY, hypodermis; MG, midgut; MUS, muscles; SG, salivary gland; TR, trachea; YK, yolk mass. Scale, 50 μm.

resided at a posterior part (a, b and c) and sometimes at a lateral part (c) of the embryo. The tissue region gave an inside-out impression, with the hypodermis accompanying muscles almost at the central part. The midgut, trachea, cephalopharyngeal apparatus, brain, salivary gland, hypodermis and muscles were recognized, but most of them were located at unordered places (the number of cells which consist of each tissue seemed to be smaller than normal). The ventral nervous system was never found in a complete form; instead, the necrotic cells (arrows) were dispersed at the surface of the embryos.

Another remarkable status found in the present study is the intercellular areas with low electron density as seen in Figure 2; these areas contained a number of peculiar structures pointed by arrows. We concluded the latter to be SROs from their form and their mode of existence. The intercellular spacing and the SRO-like structures were not detected in the specimens from normal embryos. Moreover, these curious features were very similar to those observable in the ovaries of SRO-infected *D. melanogaster* (Fig. 6 and Niki, personal communication). These may be the first electron microscopic observation of the SRO-like structures in the eggs and ovaries.

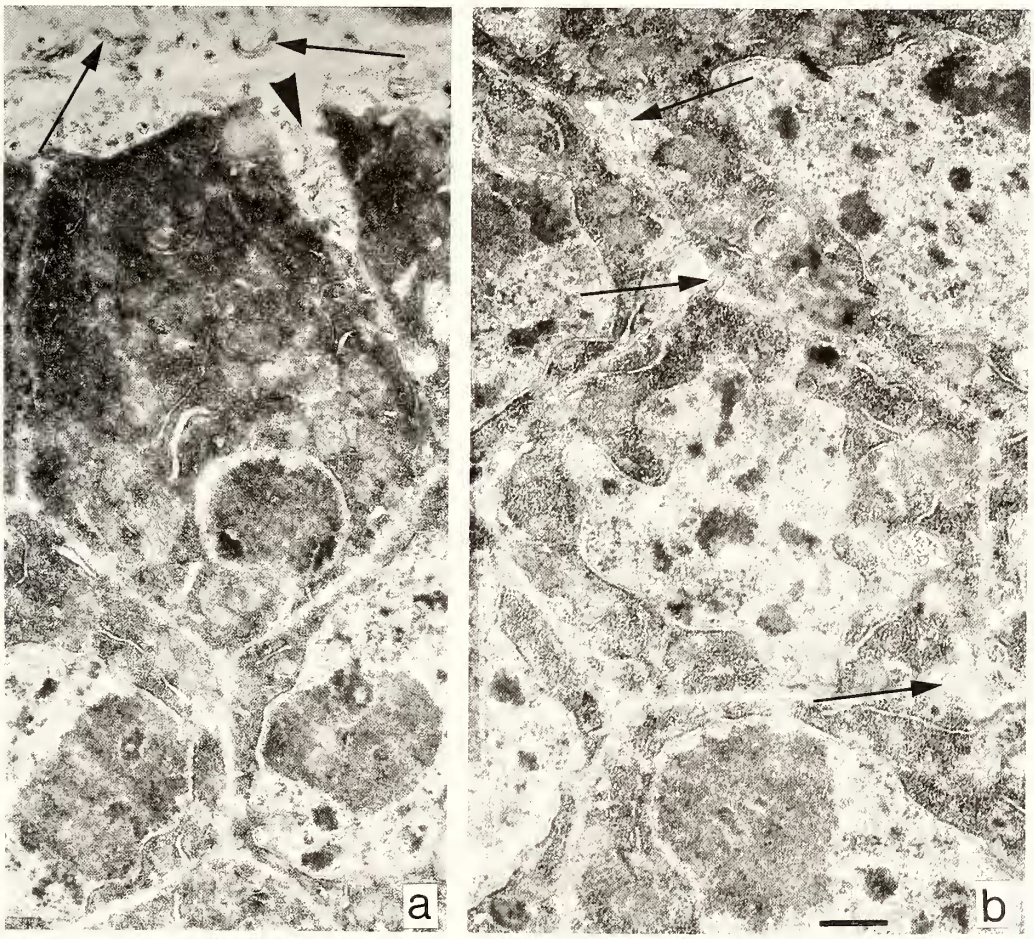

FIG. 6. Electron micrograph of parts of ovary infected with sex-ratio organisms. A female adult (Oregon-R) was dissected and the ovaries were taken. a) In upper side of the follicle cells a large number of SRO-like structures were observed (arrows) and some of them seem to invade between the follicle cells (arrowhead). b) SRO-like structures are seen in the area with low electron density between the follicle cells (arrows). Scale, 1 μm.

DISCUSSION

In the SRO-infected strain, abnormalities may occur at different stages of embryogenesis. Nevertheless, in the large majority of the lethal embryos, the ventral nervous system was found to be the most severely affected organ. The occurrence of necrotic, seemingly degenerating cells in cluster must be in close association with the SRO infection, since in normally developing embryos of the control this type of cells were only occasionally found and never in cluster. These results in *D. melanogaster* were in agreement with those of the SRO-infected *D. willistoni* embryos [9].

Most tissues other than the ventral nervous system rather seemed to follow normal course of development in the SRO-infected strain although their arrangement in an embryo was highly abnormal. The ventral nervous system was never seen in a normal assembly by itself and the constituent cells became necrotic earlier than other cells. This apparent preference of the ventral nervous tissue in sensitivity to the SRO is compatible with the results of mosaic analysis of SRO-infected *D. melanogaster* [5] which indicated that the focus of SRO-lethal action included the ventral nervous system. Not only the SRO-lethal action but also many other embryonic lethalities in *Drosophila* were shown to have their focal region at the ventral side of an embryo [10]. It should be mentioned here that our previous gynandromorph analysis has suggested the mesoderm also to be included in the target of SRO action [5] but in the present study this seemed not the case. The discrepancy may be owing to the difference in developmental stage of observation: The present study was done with embryos whereas the mosaic analysis with adults.

In addition to the major late lethal embryos, there also exist a minor group of SRO-lethal embryos, i.e. several percent of the total abnormal embryos stopped their development at the stages as early as cleavage or blastoderm, thus before the establishment of the ventral nervous system. We interpret the early mechanism of androcidal SRO action in terms of some perturbation due to the presence of the SRO. Such effects should be incurable by gene activation of the part of the hosts, since female embryos with double dosage of X-chromosome-linked genes can escape from this lethal action [4]. The plausibility of the inference may be resolved by microinjection of the SRO into normal embryos.

The late effects of SRO-embryos might be explained by assuming that the ventral nervous system of the *Drosophila* embryo requires the expression of a large number of genes for its development and organization so that it is highly sensitive to disturbance owing to the hypothetical early SRO action. In support of this assumption is a preliminary observation by Nickla *et al.* [11], who indicated that a lethal mutation brings about the abnormalities at the ventral nervous system as well as the brain, although the genes concerned are known to be active in non-nervous tissues.

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